

***In vitro* cytotoxicity induced by *Clostridium perfringens* isolate carrying a chromosomal *cpe* gene is exclusively dependent on sporulation and enterotoxin production**

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Abstract

Clostridium perfringens type A is a common source of food poisoning (FP) and non-food-borne (NFB) gastrointestinal diseases in humans. In the intestinal tract, the vegetative cells sporulate and produce a major pathogenic factor, *C. perfringens* enterotoxin (CPE). Most type A FP isolates carry a chromosomal *cpe* gene, whereas NFB type A isolates typically carry a plasmid-encoded *cpe*. *In vitro*, the purified CPE protein binds to a receptor and forms pores, exerting a cytotoxic activity in epithelial cells. However, it remains unclear if CPE is indispensable for *C. perfringens* cytotoxicity. In this study, we examined the cytotoxicity of *cpe*-harboring *C. perfringens* isolates co-cultured with human intestinal epithelial Caco-2 cells. The FP strains showed severe cytotoxicity during sporulation and CPE production, but not during vegetative cell growth. While Caco-2 cells were intact during co-culturing with *cpe*-null mutant derivative of strain SM101 (a FP strain carrying a chromosomal *cpe* gene), the wild-type level cytotoxicity was observed with *cpe*-complemented strain. In contrast, both wild-type and *cpe*-null mutant derivative of the NFB strain F4969 induced Caco-2 cell death during both vegetative and sporulation growth. Collectively, the Caco-2 cell cytotoxicity caused by *C. perfringens* strain SM101 is considered to be exclusively dependent on CPE production, whereas some additional toxins

should be involved in F4969-mediated *in vitro* cytotoxicity.

Keywords: *Clostridium perfringens*; food poisoning; Caco-2 cells; enterotoxin; cytotoxicity

1. Introduction

Clostridium perfringens, a Gram-positive spore-forming anaerobic bacterium, is a major pathogen of humans and domestic animals [1]. The virulence of *C. perfringens* is largely attributed to its toxin-producing ability and isolates of this organism are classified into five types (type A to E) based upon the production of four major toxins (α , β , ϵ , and ι) [2].

The type A strains that produce α - but not β -, ϵ -, or ι -toxin, are important cause of histotoxic infections like gas gangrene in humans [3]. Some (less than 5% of global isolates) *C. perfringens* isolates produce another important toxin named *C. perfringens* enterotoxin (CPE), which is responsible for food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) diseases such as antibiotic-associated diarrhea and sporadic diarrhea [4]. Most type A FP isolates carry a chromosomal *cpe* gene, whereas NFB type A isolates typically carry a plasmid-borne *cpe* gene [5]. *C. perfringens* type A FP consistently ranks among the most common bacterial food-borne illnesses in the USA, UK, and Japan [6-8]. On the other hand, type A strains carrying a plasmid-encoded *cpe* gene cause ~5 to 10% of all cases of human NFB GI diseases [5].

In the intestinal tract, *C. perfringens* vegetative cells sporulate and produce CPE in the mother cell compartment of sporulating cells [9, 10]. At the completion of sporulation,

the mother cell lyses, and CPE is released in the intestinal lumen [1, 11]. The released CPE then binds to enterocyte receptors, certain members of the claudin-family on the tight junction [12, 13]. The bound CPE oligomerizes and perforates the plasma membrane leading to diarrhea and abdominal cramps [14-16]. Several experimental evidences proved that GI symptoms of *C. perfringens*-associated diseases are caused by the CPE. Human volunteer feeding experiments have demonstrated that ingestion of purified CPE reproduces diarrhea and abdominal cramping [17]. In rabbit or mouse intestinal loop models, purified CPE injection mediates tissue damage and fluid accumulation in the intestine [18-20]. Sarker *et al.* demonstrated that CPE expression is necessary and sufficient for *C. perfringens* strains SM101 and F4969 to cause fluid accumulation and GI damage in a rabbit ileal loop model using the lysates of sporulating cultures of *cpe*-knock out mutants [21]. However, it remains unclear whether CPE is indispensable for bacterial cytotoxicity *in vitro* [14-16].

Recently, the significance of toxins in the induction of *in vitro* cytotoxicity has been investigated using human intestinal epithelial Caco-2 cells infected with toxin gene-harboring *C. perfringens* strains and their mutants or anti-toxin antibody [22-24]. Allaart *et al.* revealed that β 2 toxin is not involved in Caco-2 cell cytotoxicity during infection with a *cpb2*-harboring *C. perfringens* strain [22]. Agr-like quorum sensing system

and VirS/VirR two-component system in a *C. perfringens* type C isolate are essential for causing *in vitro* cytotoxicity to Caco-2 cells during infection [23, 24]. However, no studies have been conducted on *in vitro* cell cytotoxicity by *C. perfringens* type A FP and NFB strains during sporulation.

In this regard, we co-cultured wild-type and *cpe*-null mutants of *C. perfringens* FP and NFB strains with Caco-2 cells, and observed cytotoxicity during bacterial sporulation. Our co-culture experiments indicate that the essential toxin (factor) to induce *in vitro* cytotoxicity by a FP strain SM101 carrying a chromosomal *cpe* gene is different from that of a NFB strain F4969 carrying a plasmid-borne *cpe* gene.

2. Materials and Methods

2.1. Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. To achieve the sporulation, bacteria were inoculated into fluid thioglycollate (FTG; BD, Franklin Lakes, NJ) medium and incubated anaerobically for 18 h at 37°C. One milliliter of the bacterial culture was passaged into 10 ml of Duncan-Strong medium [25] and cultured for 24 h at 37°C. One milliliter of the culture was then heated at 75°C (strains NCTC8239 and F4969) or 65°C (strain SM101) for 20 min and passaged into 10 ml of fresh Duncan-Strong medium. The heat treatment and passage were repeated until the amount of spores observed by phase-contrast microscopy was greater than half of the total number of bacteria. These bacterial cells were heated and stored at -80°C with glycerol for future use.

2.2. Co-culture study. Human colonic Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. The cells were plated in a 24-well plate (1.3×10^5 cells per well) and incubated for 4 days. Just before the inoculation of bacteria, the cells were washed using phosphate buffered saline (PBS) three times and incubated in 0.65 ml of glucose-negative DMEM (DMEM(-); Life Science Technologies, Carlsbad, CA) supplemented with 0.4% glucose, 0.4% starch, or 0.4%

starch containing deoxycholate (DCA; Wako, Osaka, Japan) or ox bile (Wako). The bacterial strains prepared as described above were pre-cultured in FTG anaerobically overnight at 37°C. The cultures were washed with PBS twice and sixty-five microliters of bacterial culture (1×10^5 colony-forming units (CFU) per ml in strain SM101 or $1-5 \times 10^7$ CFU/ml in other strains) were inoculated into Caco-2 cells prepared as described above and incubated in the CO₂ incubator at 37°C. The turbidity of the cultures was monitored by measuring optical density at 650 nm (OD₆₅₀) using a spectrophotometer (V630B10; Jasco, Easton, MD).

The OD₆₅₀ for bacterial growth was calculated as follows:

$$\text{OD}_{650} (\text{bacteria}) = \text{OD}_{650} (\text{whole}) - \text{OD}_{650} (\text{Caco-2 cells}),$$

where OD₆₅₀ (whole) is an OD₆₅₀ of cell suspension containing both bacteria and Caco-2 cells, and OD₆₅₀ (Caco-2 cells) is an OD₆₅₀ of cell suspension containing only non-infected Caco-2 cells. The number of viable vegetative cells was determined by plating serially diluted samples onto Brain Heart Infusion (BHI) agar, incubating at 37°C for 24 h in anaerobic conditions, and calculating CFU. The number of heat-resistant spores was counted by plating heat-treated cultures onto BHI agar. The detection threshold was 200 CFU/ml.

2.3. Western blotting. Cultures were centrifuged at $2,350 \times g$ for 10 min and supernatants

(20 μ l) were subjected to 12% SDS-PAGE. After transferring to a PVDF membrane, the samples were probed with a rabbit anti-CPE antibody kindly provided by Dr. Yasuhiko Horiguchi (Osaka University, Japan) or a rabbit anti- α -toxin antibody kindly provided by Dr. Masahiro Nagahama (Tokushima Bunri University, Japan) for 1 h at 37°C. A horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was then reacted for 1 h at 37°C and detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK).

2.4. Caco-2 cell cytotoxicity assay. The LDH cytotoxicity test was performed [26] according to the manufacturer's instructions (Wako) with slight modifications. Briefly, co-cultures of *C. perfringens*/Caco-2 cells were centrifuged at 150 \times g for 3 min. Supernatant (12.5 μ l) was transferred to a 96-well assay plate and diluted with 37.5 μ l of distilled water. Samples cultured in DMEM(-) with 0.4% starch and 0.1% Tween 20 were used as positive controls. The supernatants of non-infected Caco-2 cells were used as negative controls. After 50 μ l of reactive solution was dispensed into each well, the plate was incubated for 15 min at room temperature. After termination of the reaction with 100 μ l of stop solution, the absorbance of each sample was measured at 560 nm in an automated plate reader (ARBO X5; PerkinElmer,

Waltham, MA). The cell injury rate was calculated according to the manufacturer's instructions.

2.5. RNA extraction. Bacterial cultures containing 2×10^7 CFU of *C. perfringens* were treated with RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After centrifugation at $5,000 \times g$ for 10 min, the pellets were washed with SET buffer (25% sucrose, 50 mM EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0)) at $5,000 \times g$ for 10 min. The pellets, which were suspended in GTC buffer (4 M guanidine thiocyanate, 0.5% Na N-lauryl sarcosine, 25 mM sodium citrate (pH 7.0) and 0.1 M β -mercaptoethanol) [27, 28], were homogenized by passing 3 times through a 21-gauge needle to disrupt Caco-2 cell membranes. After being centrifuged at $5,000 \times g$ for 10 min, the pellets were washed with SET buffer once. The bacterial cells were lysed by suspending in 100 μ l SET buffer with 20 mg/ml lysozyme (Sigma) and 100 μ g/ml proteinase K (Roche Applied Science, Upper Bavaria, Germany) at 37°C for 30 min. Following incubation, they were transferred to a tube containing zirconia beads (Easy Beads; AMR, Gifu, Japan), vortexed for 5 min, and centrifuged at $21,130 \times g$ for 5 min. Total RNA was extracted from the supernatants using TRI reagent LS (Sigma) according to the manufacturer's instruction.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was digested with DNase I (RQ1 RNase-Free DNase; Promega, Madison, WI) according to the manufacturer's instructions. cDNA was synthesized using Superscript III reverse transcriptase (Life Science Technologies) with the random primers (Life Science Technologies). Synthesized cDNA was subjected to PCR using Go Taq DNA polymerase (Promega) with gene-specific forward and reverse primer sets (Table 2).

2.7. DNA extraction. Genome DNA from *C. perfringens* was purified from overnight cultures grown in GAM broth (Nissui, Tokyo, Japan) [29]. Briefly, after 2 ml cultures were centrifuged at $21,130 \times g$ for 5 min, the pellets were suspended in 400 μ l TES buffer (50mM Tris-HCl (pH 8.0), 1mM EDTA, and 6.7% sucrose) and incubated for 5 min at 37°C. After addition of 100 μ l of 20 mg/ml lysozyme and incubation for 10 min at 37°C, they were mixed with 160 μ l Tris-EDTA buffer (50mM Tris-HCl (pH 8.0) and 0.25 M EDTA) containing 20% SDS and incubated for 15 min at 37°C. Then, 20 μ g RNase (NipponGene, Tokyo, Japan) was added, and the samples were incubated for 15 min at room temperature. DNA was extracted by phenol:chloroform:isoamyl alcohol (Nakarai, Kyoto, Japan) and precipitated by

isopropanol.

2.8. Southern blotting. Purified DNA samples were digested overnight with *HindIII* or *XbaI* at 37°C according to the manufacturer's instructions (Takara, Shiga, Japan) and separated by electrophoresis on a 1% agarose gel. The separated DNA digestion products were transferred onto a positively charged nylon membrane (Hybond-N+; GE Healthcare) for hybridization with a digoxigenin (DIG)-labeled intron-specific probe, which was prepared as previously described [30] using the primer pair PrMY8 and 9 (Table 2). CSPD substrate (Roche Applied Science) was used to detect probe hybridization according to the manufacturer's instructions.

2.9. Construction of a *C. perfringens* strain SM101 *cpe*-null mutant. The *cpe* gene of SM101 was inactivated by insertion of a group II intron using the *Clostridium*-modified TargeTron (Sigma) insertional mutagenesis system [31, 32]. Utilizing optimal intron insertion sites identified in the SM101 genome sequence on the Sigma TargeTron website, an intron was targeted in the antisense orientation between nucleotides 195 and 196 of the *cpe* ORF. The primers used to target the intron to the *cpe* ORF were PrMY8, 9, 10, and 17 as shown in Table 2. A 350 bp PCR product was inserted into pJIR750ai to construct a

cpe-specific TargeTron plasmid. The resultant plasmid, named pMY4, was electroporated into wild-type SM101 by MicroPulser (Bio-Rad, Hercules, CA) (1,500 V, 4 mS). Transformants were selected onto BHI agar plates containing 15 µg/ml of chloramphenicol. Bacterial clones carrying an intron insertion were screened by PCR using primers PrMY20, 21, and 3 as shown in Table 2.

2.10. Construction of complementing strains for *cpe*-null mutants. The *cpe*-null mutant MY15 was passaged in BHI broth to cure the plasmid, and confirmed the deletion of the plasmid by sensitivity to chloramphenicol. The *cpe* expression plasmid pJRC200 [33] was then electroporated into strain MY15 by MicroPulser. Transformants were selected onto BHI agar plates containing 50 µg/ml of erythromycin.

2.11. Reverse passive latex agglutination assay. The assay was performed according to the manufacturer's information (PET-RPLA; Denka Seiken Co., Ltd, Niigata, Japan). Briefly, co-cultures of *C. perfringens*/Caco-2 cells were centrifuged at $900 \times g$ for 20 min. The supernatants were serially 4-fold diluted in a 96-well plate, and latex was added. The plate was then incubated at room temperature for 18 h.

2.12. Transepithelial electrical resistance (TER). Caco-2 cells were seeded on collagen-coated permeable membrane supports (Transwell-COL; Corning Incorporated, Corning, NY) placed in 24-well plates at a density of 7.5×10^4 cells per well, and incubated for 4 days in a CO₂ incubator. *C. perfringens* were co-cultured with Caco-2 cells as described above but with slight modifications. Bacterial suspension (150 µl) was added to the apical compartment of the Transwell chamber. The TER across the monolayer was measured with Millicell-ERS (Merck, Darmstadt, Germany) [34].

2.13. Statistical analyses. Data are expressed as means \pm SD or SEM. Statistical analysis was performed by Student's or Welch's *t*-test. The statistical significance of multiple comparisons was determined by one-way ANOVA, followed by the Tukey–Kramer test. *P* < 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of *C. perfringens* strains during vegetative cell growth. *C. perfringens* is an anaerobic bacterium that generally grows in oxygen-limiting conditions. To create conditions suitable for bacterial growth and its pathogenic effect on host cells in a single experiment, we co-cultured *C. perfringens* strains (NCTC8239, W5837, or W09-505 derived from FP, or JCM1290 derived from gas gangrene) with human intestinal Caco-2 cells in DMEM(-) containing 0.4% glucose (G/DMEM(-)) in a CO₂ incubator. In the presence of Caco-2 cells, robust bacterial growth was observed with all the strains tested at 8 h post-inoculation (hpi) (Fig. 1A), implying that the metabolism of Caco-2 cells, such as consumption of oxygen and/or secretion of metabolites, is suitable to *C. perfringens* cell growth. During bacterial growth, sporulation and production of CPE were not observed for all the strains (Figs. S1A and S1B). To measure cytotoxicity, we first confirmed that *C. perfringens* did not release detectable LDH by using the supernatants of the cultured bacteria in the absence of Caco-2 cells (data not shown). Caco-2 cells were not damaged by the FP strains during the experiment, but the gas gangrene strain JCM1290 showed profound cytotoxicity at 8 hpi (Fig. 1B). These results indicate that the *C. perfringens* FP strains do not induce cytotoxicity to Caco-2 cells during vegetative cell growth.

3.2. Production of toxins by *C. perfringens* strains during vegetative cell growth.

To understand the mechanism of JCM1290-mediated cytotoxicity during vegetative cell growth, we assessed the production of α -toxin and the expression of *pfoA* gene encoding perfringolysin O. When *C. perfringens* isolates were co-cultured with Caco-2 cells in G/DMEM(-), α -toxin was detected in the supernatants of strain JCM1290 at 4 hpi by Western blot analyses (Fig. 1C). In contrast, all tested FP strains did not produce detectable α -toxin. Our PCR survey failed to detect *pfoA* gene in FP strains tested (Fig. S2), which is consistent with the previous finding that most of the type A chromosomal *cpe* strains do not carry *pfoA* [35]. When the *pfoA* mRNA expression level was determined in strain JCM1290 co-cultured with Caco-2 cells in G/DMEM(-), the expression of *pfoA* was increased at 4 hpi compared to 0 hpi, and then decreased at 8 hpi (Fig. 1D). No *pfoA*-specific band was detected in PCR without reverse transcription (data not shown). These results suggest that the α -toxin and perfringolysin O are likely to play some role in the Caco-2 cell cytotoxicity by strain JCM1290 during vegetative cell growth.

3.3. Cytotoxicity of a *C. perfringens* FP strain during sporulation.

we used starch [36] instead of glucose as a carbon source in the co-culture medium because glucose is known to inhibit sporulation [37, 38]. A bile salt (DCA), previously reported to increase sporulation [39-41], was also used. We confirmed that 0.001–0.1% ox bile and 0.4–250 μ M DCA indeed accelerated sporulation of strain NCTC8239 in our co-culture system (Figs S3A and S3B). When we co-cultured strain NCTC8239 in G/DMEM(-), DMEM(-) containing 0.4% starch (S/DMEM(-)), or DMEM containing 0.4% starch and 50 μ M DCA (S/D/DMEM(-)), the turbidity of the cultures increased significantly in the presence of Caco-2 cells (Fig. 2A), indicating that these media supported bacterial growth similarly. As expected, sporulation was not detected in G/DMEM(-) (Fig. 2B). By contrast, sporulation was induced in S/DMEM(-); the number of spores were 1×10^3 and 2×10^4 CFU/ml at 8 and 24 hpi, respectively. The addition of DCA further induced sporulation, and the number of spores reached 1×10^6 CFU/ml at 8 hpi. Increased production of CPE was also observed in the presence of DCA or bile at 24 hpi (Figs 2C, S3C, and S3D). We then assessed cytotoxicity quantitatively in Caco-2 cells using the LDH assay. We first confirmed that all the media used in this study were not cytotoxic in the LDH assay in the absence of bacteria (data not shown). Strain NCTC8239 cultured in G/DMEM(-) did not induce cytotoxicity until 24 hpi (Fig. 2D). By contrast, cell death was observed in NCTC8239 cultured in S/DMEM(-),

and exacerbated when it was cultured in S/D/DMEM(-) at 24 hpi (Fig. 2D). Addition of bile also increased the cytotoxicity of NCTC8239 (Fig. S3E). Collectively, these results suggest that the cytotoxicity by strain NCTC8239 during co-culturing in S/DMEM(-) and S/D/DMEM(-) was due to the induction of sporulation and production of CPE.

3.4. Cytotoxicity of *cpe*-null mutant during sporulation. To confirm whether the cytotoxicity of the *C. perfringens* FP strain is mediated by the production of CPE during sporulation, we introduced a *cpe*-null mutation in strain SM101 (a derivative of FP strain NCTC8798) using the Targetron method [11]. The mutator plasmid pMY4 designed to insert intron to the *cpe* gene (Fig. 3A) was introduced into SM101 by electroporation and the *cpe*-null mutant strain MY15 was isolated as previously described [32]. The PCR analyses confirmed the insertion of intron into the *cpe* gene in MY15 (Fig. 3B). Southern blot analysis using an intron-specific probe demonstrated the presence of a single intron insertion into the genome of the *cpe*-null mutant MY15 (Fig. 3C). Complemented MY15 was prepared by introducing the recombinant plasmid pJRC200 [33] carrying wild-type *cpe* into MY15 by electroporation. The presence of wild-type *cpe* (Fig. 3B, upper panel) and the insertion of intron (Fig. 3B, lower panel) were confirmed in MY15(pJRC200) by the PCR analysis. The

fragment with an intron insertion (1.1 kb band in Fig. 3B, upper panel) was not detected in MY15(pJRC200), suggesting that multiple copies of the *cpe* gene of plasmid origin, whose amplification far outweighs that of the mutated chromosomal *cpe*, are present in MY15(pJRC200). When strains SM101, MY15, and MY15(pJRC200) were co-cultured with Caco-2 cells in S/D/DMEM(-) for 36 h, CPE was detected in the culture supernatants with SM101 and MY15(pJRC200), but not with MY15, by Western blot analysis (Fig. 3D). These results indicated that strain MY15 is defective in CPE production and this defect is restored in the complemented strain MY15(pJRC200).

We then co-cultured SM101, MY15, or MY15(pJRC200) with Caco-2 cells in S/D/DMEM(-) and assessed bacterial growth and sporulation. Strains SM101 and MY15 showed similar kinetics in their growth and sporulation: the numbers of vegetative and sporulating cells each reached 1×10^6 CFU/ml at 12 hpi and then gradually decreased until 36 hpi (Fig. 4A). CPE was detected at 12 hpi in the culture supernatants of SM101 by Western blot analysis (Fig. 4B). As expected, MY15 did not produce CPE during the co-culture experiment. When MY15(pJRC200) was co-cultured with Caco-2 cells at 1×10^4 CFU/ml (the equivalent titer to SM101 and MY15), it showed lower bacterial growth (1×10^5 CFU/ml at 12 hpi) and sporulation (1×10^4 CFU/ml at 24 hpi) compared to SM101 (Fig.

S4A). MY15(pJRC200) restored the production of CPE but it was detectable at 36 hpi (Fig. S4B). In order to detect enough amounts of spores and CPE in the cultures of MY15(pJRC200), we co-cultured MY15(pJRC200) at 1×10^5 CFU/ml. In those, the number of spore cells reached 1×10^5 CFU/ml and the detectable amount of CPE was restored at 12 hpi (Figs 4A and 4B). Therefore, MY15(pJRC200) was co-cultured at 1×10^5 CFU/ml for further research. To measure the concentration of CPE released in the culture, we performed Western blot analysis using the 20 μ l supernatant of SM101 at 12 hpi and compared the intensity of the band of CPE to those in 1 to 1,000 ng CPE (Fig. 4C). The intensity of the band of CPE in SM101 was equivalent to that in 100 ng CPE. Therefore the amount of CPE in the supernatants of SM101 was estimated to be approximately 5 μ g/ml CPE at 12 hpi. We next employed a reverse passive latex agglutination assay to detect CPE at 3, 6, 9, and 12 hpi with SM101 (Fig. 4D) because it was predictable that the amount of CPE produced was not detectable in Western blot analysis in the earlier phase of co-culture. The latex agglutination titers were between 2 and 8 in the supernatants of 3 and 6 hpi, and increased to 512 to 8,192 at 9 and 12 hpi. The titers at 12 hpi were equivalent to those of controls: 10 μ g/ml CPE. These results indicate that the amount of CPE gets increased in the supernatants at 6 to 9 hpi and reaches 5 to 10 μ g/ml at 12 hpi in the co-culture condition.

We next assessed the cytotoxicity on Caco-2 cells by these strains in *C. perfringens*/Caco-2 co-cultures at 12, 24, and 36 hpi by phase-contrast microscopy (Fig. 5A). Caco-2 cells co-cultured with SM101 showed cell rounding and detachment at 12 hpi. By contrast, MY15 did not show any cytotoxic effect until 36 hpi. MY15(pJRC200) restored cytotoxicity, and Caco-2 cells were damaged at 12 hpi as in SM101. Consistent with these observations, LDH release from the cells significantly increased with SM101 at 12 hpi; the rate of cytotoxicity was approximately 50% at 12 hpi and reached 80% at 24 hpi (Fig. 5B). Cytotoxicity was not detected with MY15 until 24 hpi and then slightly increased at 48 hpi. As expected, MY15(pJRC200) restored LDH release, and the cytotoxicity reached similar levels as that of SM101 at 12 hpi. We also assessed the epithelial cell integrity by measuring the TER [42] across the Caco-2 monolayer (Fig. 5C) because CPE is known to bind claudins, components of tight junctions, and disrupt tight junction barriers [43]. The TER decreased in the SM101 co-culture at 4 hpi and significantly reduced until 10 hpi. By contrast, MY15 did not induce any TER decrease during the experiment, as observed with non-infected cells. MY15(pJRC200) restored the significant decrease in TER. MY15(pJRC200) co-cultured at 1×10^4 CFU/ml also showed cytotoxicity by the LDH assay at 36 hpi (Fig. S4C). The delay of cytotoxicity is consistent with the delayed release of CPE (Figs S4A and S4B). On the other

hand, MY15(pJRC200) co-cultured at 1×10^4 CFU/ml showed remarkable decrease in the TER as that in SM101 (Fig. S4D), suggesting that the TER is more sensitive assay to assess Caco-2 cell damage than the LDH assay. Collectively, *C. perfringens* FP strain SM101 shows a cytotoxicity to Caco-2 cells that is exclusively dependent on the production of CPE during sporulation.

3.5. Cytotoxicity of a *C. perfringens* NFB strain. Most type A FP strains encode the *cpe* gene in their chromosome, whereas NFB strains typically carry a plasmid-borne *cpe* gene. We therefore examined whether a *C. perfringens* NFB strain F4969 carrying a plasmid-borne *cpe* gene shows CPE-dependent cytotoxicity on Caco-2 cells like FP strain SM101. First, we co-cultured strain F4969 or its *cpe*-null mutant MRS4969 with Caco-2 cells in G/DMEM(-) or S/D/DMEM(-), and assessed bacterial cell growth and sporulation (Fig. 6A). The number of vegetative cells was approximately 1×10^6 CFU/ml at 8 and 24 hpi in both strains cultured in G/DMEM(-) or S/D/DMEM(-). As expected, no sporulating cells were detected in strain F4969 cultured in G/DMEM(-). By contrast, strains F4969 and MRS4969 sporulated similarly in S/D/DMEM(-); the number of spores reached approximately 1×10^5 CFU/ml at 8 hpi. We next examined cytotoxicity of the strains (Fig. 6B). LDH release occurred at 8 hpi in

strain F4969 in G/DMEM(-). We also observed cytotoxicity of strain F4969 during sporulation in S/D/DMEM(-) at 8 hpi. It is noteworthy that the LDH release was observed with *cpe*-null mutant strain MRS4969 similarly to wild-type F4969 during sporulation at 8 and 24 hpi. In order to dissect the role of toxins for cytotoxicity by strain F4969, we assessed the production of CPE and α -toxin, and the expression of *pfoA* gene. When the supernatants of the strains co-cultured with Caco-2 cells were subjected to Western blot analysis, CPE was not detected in F4969 cultured in G/DMEM(-) during the experiment (Fig. 6C, upper panel). During sporulation in S/D/DMEM(-), detectable CPE was produced at 24 hpi by strain F4969 but not strain MRS4969 as expected. In Western blot analysis, both strains F4969 and MRS4969 produced detectable amount of α -toxin at 8 and 24 hpi in S/D/DMEM(-) (Fig. 6C, lower panel). By contrast, α -toxin was detected slightly at 8 hpi in F4969 in G/DMEM(-). Finally when we examined the expression levels of the *pfoA* by RT-PCR (Fig. 6D), strain F4969 cultured in both G/DMEM(-) and S/D/DMEM(-) expressed the *pfoA* gene higher at 4 and 8 hpi than 0 hpi. Strain MRS4969 also increased the expression level of the *pfoA* gene at 4 hpi compared to 0 hpi. No *pfoA*-specific band was detected in PCR without reverse transcription (data not shown). Collectively, the results suggest that the Caco-2 cell

cytotoxicity caused by the NFB strain F4969 is not dependent on CPE production, and perhaps other toxin(s) plays a major role to induce this cytotoxicity.

4. Discussion

Previous *in vivo* study demonstrated that CPE expression is both necessary and sufficient for *C. perfringens* type A disease isolates SM101 and F4969 to induce histopathological damage in rabbit ileal loop model [21]. *In vitro*, purified CPE has showed host cell cytotoxicity by forming pores on the surface of cell membrane [12, 14-16]. However, it is still unknown whether CPE is indispensable for *in vitro* host cell cytotoxicity. In the current study, we demonstrated that CPE is essential to induce *in vitro* cytotoxicity caused by *C. perfringens* FP strain SM101 harboring a chromosomal *cpe* by co-culturing *cpe*-null mutants with Caco-2 cells. By contrast, CPE does not play a major role to induce *in vitro* cytotoxicity by NFB strain F4969 carrying a plasmid-encoded *cpe*, as *cpe*-null mutant derivative of F4969 (strain MRS4969) showed cytotoxicity to Caco-2 cells during co-culture. It was unexpected because cell lysates of both strains, SM101 and F4969, caused CPE-dependent fluid accumulation and histopathological damage in a rabbit ileal loop model [21]. These discrepancies suggest that some additional toxins might be responsible for F4969-mediated *in vitro* cytotoxicity. Sayeed *et al.* showed that perfringolysin O and α -toxin are not essential for the intestinal virulence of *C. perfringens* type C isolate CN3685 in a rabbit ileal loop model using the *cpb*, *pfoA*, or *plc*-null mutants [44]. On the other hand,

perfringolysin O was reported to be the most responsible toxin for the *C. perfringens* strain 13-dependent *in vitro* cytotoxicity [45]. Indeed, our current study demonstrated that during co-culturing with Caco-2 cells, (i) NFB strain F4969 expressed the *pfoA* mRNA (Fig. 6D), whereas FP strains do not even carry the *pfoA* gene (Fig. S2); and (ii) strain F4969 produced detectable level of α -toxin in the sporulation condition (Fig. 6C). Thus, perfringolysin O and α -toxin (and perhaps other toxins) are likely to be more important than CPE for *in vitro* cytotoxicity induced by strain F4969. However, it is still unclear (i) which bacterial factor(s) is essential to induce *in vitro* cytotoxicity in strain F4969, (ii) whether most, if not all, isolates derived from FP or NFB diseases reproduce the results for *in vitro* cytotoxicity shown in this study, and (iii) whether the difference in *in vitro* cytotoxicity between a FP and NFB strain influences in *in vivo* pathogenicity, even though rabbit ileal loop model revealed that CPE is an essential factor for the GI damage [21]. Further studies employing a large number of FP and NFB strains and specific gene-deficient isogenic mutants should clarify these unanswered questions.

The higher production of α -toxin by a gas gangrene strain JCM1290 during co-culture, compared to FP strains (Fig. 1C), suggested that the regulation of α -toxin production in strain JCM1290 might be different from that in the tested FP strains. *plc*

expression is positively regulated by VirR/VirS through VR-RNA [46, 47]. Recently, Obana *et al.* reported that VR-RNA matures and activates mRNA of *colA*, another toxin regulated by VirR/VirS/VR-RNA, by pairing to the sequence of 5'UTR of *colA* [48, 49]. Also, the production of α -toxin is partly dependent on the promoter sequence of *plc* [50]. Indeed, the promoter sequence of *plc* in strain NCTC8239 is identical with that of strain 13 that produces intermediate level of α -toxin. In contrast, strain JCM1290 *plc* promoter is identical to that of strain NCTC8237 producing high level of α -toxin. Recently, Katayama *et al.* described that the specific affinity of the *C. perfringens* polymerase subunits for the phased A-tracts upstream of the *plc* promoter is likely to contribute to gene expression [51]. Although the molecular mechanism of the regulation of toxin genes is not fully understood in *C. perfringens*, it is possible that the promoter sequences of toxin genes and the affinity of regulatory factors and polymerases to the binding regions would influence to the abilities of toxin production, leading to different Caco-2 cell cytotoxicity in *C. perfringens*.

In the present study, we developed a *C. perfringens*/Caco-2 co-culture system where the anaerobic *C. perfringens* grew and exerted its pathogenic effects on host cells in a CO₂ incubator. This model system enables us to obtain reproducible results without any laborious regulation of oxygen content in the culture conditions. Modification of medium

constituents is also easy, which allows us to control the growth condition for vegetative and sporulating cells. Indeed, *C. perfringens* cells grew to a level of 10^6 CFU/ml, formed spores extensively, and produced several micrograms of CPE in the presence of Caco-2 cells in a single culture well. Also 5–10 $\mu\text{g/ml}$ of CPE released during sporulation was sufficient to cause cytotoxicity by strain SM101 in this system. This is consistent with the previous reports in which 10–50 $\mu\text{g/ml}$ of CPE caused fluid accumulation and damage to intestinal villi in animal intestinal loop models and human ileum tissues [18-20, 52]. Recent studies revealed host-pathogen cross-talk between *C. perfringens* and the cultured host cells including Caco-2 cells; *C. perfringens* vegetative cells significantly up-regulate the production of many toxins when grown in the presence of cultured Caco-2 cells [53], and Agr-like quorum sensing and VirS/VirR systems participate in this signaling [23, 24, 54]. Although it has not yet been determined whether host-pathogen cross-talk is also applicable for CPE-positive type A strains, our *in vitro* co-culture model system can be used to assess the complicated interactions between environmental factors, bacterial cells, and host cells that usually take place within patients' intestinal tracts [9].

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Figure legends

Fig. 1. Cytotoxicity during *C. perfringens* cell growth. (A) *C. perfringens* strains were cultured with or without Caco-2 cells in a 24-well plate in G/DMEM(-) for 8 or 24 h in a CO₂ incubator and turbidity was measured. **: $P < 0.01$ compared to absence of Caco-2 cells. Data represent the means + SD of three independent experiments. (B) Strain JCM1290 (solid diamonds), NCTC8239 (open diamonds), W5837 (solid squares), or W09-505 (open squares) were co-cultured with Caco-2 cells in a 96-well plate in G/DMEM(-) for 8 or 24 h, and cytotoxicity was measured by the LDH assay. **: $P < 0.01$ compared to other strains. Data represent the means \pm SEM of three independent experiments. (C) *C. perfringens* strains were co-cultured with Caco-2 cells in a 24-well plate in G/DMEM(-) for 4 or 24 h. The supernatants were subjected to Western blot analysis using an α -toxin specific antibody. The supernatant of strain NCTC8239 cultured in TGY broth for 24 h was used as a positive control (P). (D) Strain JCM1290 was co-cultured with Caco-2 cells in G/DMEM(-). At 0, 4, or 8 hpi, the samples were collected, and total RNA was extracted. After DNase and RT treatment, mRNA expression of *pfoA* (upper panel) was determined by PCR. 16S rRNA (lower panel) indicates an internal control. M indicates molecular size marker.

Fig. 2. Cytotoxicity of a *C. perfringens* FP strain during sporulation. Strain NCTC8239 was co-cultured with Caco-2 cells in G/DMEM(-) (diamonds), S/DMEM(-) (squares), or S/D/DMEM(-) (triangles) for 8 or 24 h in a CO₂ incubator. (A) The turbidity of NCTC8239 was measured in the presence (solid symbols) or absence (open symbols) of Caco-2 cells. **: $P < 0.01$ compared to absence of Caco-2 cells. Data represent the means \pm SD of three independent experiments. (B) The number of vegetative cells (solid symbols) or spores (open symbols) was determined by plating serially diluted unheated (for vegetative cells) or heat-treated (for heat-resistant spores) samples, respectively, onto BHI agar, incubating at 37°C for 24 h in anaerobic conditions and counting CFU. Data represent the means \pm SEM of three independent experiments. (C) The presence of CPE in the supernatants of the co-cultures at 24 hpi was assessed by Western blot analyses using a CPE-specific antibody. M indicates molecular size marker. (D) The cytotoxicity of Caco-2 cells was measured by LDH assay. *: $P < 0.05$ compared to G/DMEM(-). Data represent the means \pm SEM of three independent experiments.

Fig. 3. Construction of *cpe*-null mutant of strain SM101. (A) A schematic diagram showing the location of intron insertion, primer target regions (PrMY3, 20, and 21) used in

Fig. 3B, and recognition sites of *HindIII* and *XbaI*. (B) A single colony from wild-type SM101, *cpe* mutant MY15, and complemented MY15(pJRC200) was subjected to PCR using the *cpe*-specific primers PrMY20 and PrMY21 (upper panel) or *cpe*-intron primers PrMY20 and PrMY3 (lower panel). M indicates molecular size marker. (C) Southern hybridization analysis for the presence of a group II intron insertion in SM101 and MY15. Genomic DNA from each strain was digested with *HindIII* or *XbaI*, and electrophoresed on a 1% agarose gel. After transferred onto a nylon membrane, it was hybridized with a DIG-labeled intron-specific probe. (D) SM101, MY15, and MY15(pJRC200) were co-cultured with Caco-2 cells in S/D/DMEM(-) for 36 h. Culture supernatants were subjected to Western blot analyses using a CPE-specific antibody.

Fig. 4. Kinetics of bacterial growth and production of CPE by strain SM101 and its *cpe*-null mutant. Strain SM101 (diamonds), MY15 (squares), or MY15(pJRC200) (triangles) was co-cultured with Caco-2 cells in S/D/DMEM(-). (A) The number of vegetative cells (solid symbols) or heat-resistant spores (open symbols) was measured by CFU at 0, 12, 18, 24, and 36 hpi. Data represent the means \pm SEM of three independent experiments. (B) The supernatants of the *C. perfringens*/Caco-2 co-culture samples at 0, 12,

18, 24, and 36 hpi were subjected to Western blot analyses using a CPE-specific antibody. (C) The supernatant of SM101/Caco-2 co-culture for 12 h was subjected to Western blot analysis with purified 1, 10, 100, and 1,000 ng CPE. The presence of CPE was detected by a CPE-specific antibody. M indicates molecular size marker. (D) Strain SM101 was co-cultured with Caco-2 cells, and samples at 3, 6, 9, and 12 hpi were subjected to a reversed passive latex agglutination assay for detection of CPE. C indicates 10 µg/ml CPE. The experiments were performed three times independently.

Fig. 5. Cytotoxicity of strain SM101 and its *cpe*-null mutant. Strain SM101 (diamonds), MY15 (squares), or MY15(pJRC200) (triangles) was co-cultured with Caco-2 cells in S/D/DMEM(-). (A) Caco-2 cells were observed at 12, 24, and 36 hpi by phase-contrast microscopy. Control indicates non-infected cells. Scale bar is 50 µM. (B) The bacterial strains were co-cultured in a 96-well microtiter plate, and the cytotoxicity was measured by LDH assay at 12, 24, 36, and 48 hpi. *: $P < 0.05$, **: $P < 0.01$ compared to MY15. Data represent the means \pm SEM of three independent experiments. (C) The bacterial strains were co-cultured with Caco-2 cells in Transwells. TER was measured at 0, 2, 4, 6, 8, and 10 hpi. The y-axis indicates the rate of TER values against those at 0 hpi. The solid gray line

represents TER values in non-infected cells. **: $P < 0.01$ compared to non-infected cells and MY15. Data represent the means \pm SD of three independent experiments.

Fig. 6. Cytotoxicity of the *C. perfringens* NFB strain F4969 and its *cpe*-null mutant.

Strain F4969 carrying a plasmid-borne *cpe* gene or its *cpe*-null mutant MRS4969 was co-cultured with Caco-2 cells in G/DMEM(-) for vegetative cell growth or S/D/DMEM(-) for sporulation for 4, 8 or 24 h. (A) The number of vegetative cells (solid symbols) or spores (open symbols) was determined by CFU in F4969 in G/DMEM(-) (diamonds), F4969 in S/D/DMEM(-) (squares), and MRS4969 in S/D/DMEM(-) (triangles). Data represent the means \pm SEM of three independent experiments. (B) The bacterial strains were co-cultured as in Fig. 6A in a 96-well microtiter plate, and the cytotoxicity was measured by LDH assay. Data represent the means \pm SEM of three independent experiments. (C) The cultured supernatants were subjected to Western blot analysis using a CPE (upper panel) or an α -toxin (lower panel) specific antibody. (D) Co-cultured samples were collected at 0, 4, or 8 hpi, and total RNA was extracted. After DNase and RT treatment, mRNA expression of *pfoA* (upper panel) was determined by PCR. 16S rRNA (lower panel) indicates an internal control. M indicates molecular size marker.

Supporting information

Fig. S1. Sporulation and CPE production by *C. perfringens* strains in G/DMEM(-). *C. perfringens* strains were co-cultured as in Fig. 1A. (A) The number of vegetative cells (solid bars) and spores was determined by CFU at 8 or 24 hpi. Data represent the means + SEM of three independent experiments. Asterisks indicate that the number of bacterial cells is less than the detection threshold. (B) The identical samples to Fig. 1C were subjected to Western blot analysis using a CPE-specific antibody. The supernatant of strain NCTC8239 co-cultured in S/D/DMEM(-) for 24 h is served as a positive control (P).

Fig. S2. Detection of *pfoA* gene in *C. perfringens*. *C. perfringens* strains were cultured in GAM broth for 24 h. After centrifugation, bacterial cells were suspended in distilled water and boiled. The collected supernatants were subjected to PCR using *pfoA*-specific primer sets shown in Table 2. M indicates molecular size marker.

Fig. S3. Effect of bile or bile salts on sporulation, production of CPE, and cytotoxicity by *C. perfringens*. Strain NCTC8239 was co-cultured with Caco-2 cells in G/DMEM(-), S/DMEM(-), or S/DMEM(-) with several concentrations of bile (A, C, and E) or DCA (B and

D) in a CO₂ incubator. (A) (B) The number of vegetative cells (solid bars) and spores (gray bars) was determined by CFU at 8 (A) or 24 (B) hpi. Open bars represent the sum of the numbers of vegetative cells and spores. Data represent the means + SEM of three independent experiments. (C) (D) The presence of CPE in the supernatants of the co-cultures at 24 hpi was assessed by Western blot analyses using a CPE-specific antibody. (E) The cytotoxicity of Caco-2 cells was measured by LDH assay at 4, 8, 12, and 24 hpi. G/DMEM(-) (solid diamond); S/DMEM(-) (open diamond); S/DMEM(-) with 0.005% bile (solid square); S/DMEM(-) with 0.05% bile (open square). *: $P < 0.05$, **: $P < 0.01$ compared to G/DMEM(-), and ***: $P < 0.01$ compared to all other groups. Data represent the means ± SEM of three independent experiments.

Fig. S4. Growth and cytotoxicity of *cpe*-complemented strain. Strain MY15(pJRC200) was co-cultured with Caco-2 cells in S/D/DMEM(-) at 1×10^4 CFU/ml. (A) The number of vegetative cells (solid symbols) or heat-resistant spores (open symbols) was measured by CFU at 0, 12, 18, 24, and 36 hpi. Data represent the means ± SEM of three independent experiments. (B) The supernatants of the MY15(pJRC200)/Caco-2 co-culture samples at 0, 12, 18, 24, and 36 hpi were subjected to Western blot analyses using a CPE-specific antibody.

(C) Strain MY15(pJRC200) was co-cultured in a 96-well microtiter plate, and the cytotoxicity was measured by LDH assay at 0, 6, 12, 24, 36, and 48 hpi. Data represent the means \pm SEM of three independent experiments. (D) Strain MY15(pJRC200) was co-cultured with Caco-2 cells in Transwells. TER was measured at 0, 2, 4, 6, 8, and 10 hpi. The y-axis indicates the rate of TER values against those at 0 hpi. The solid gray line represents TER values in non-infected cells. Data represent the means \pm SD of three independent experiments.

Tables

Table 1. Bacterial strains

Strains	Description	Production of CPE	Reference or source
JCM1290	Type A gas gangrene strain	–	JCM ¹
NCTC8239	Type A FP strain, carries chromosomal <i>cpe</i> gene	+	NCTC ²
W5837	Type A FP strain	+	TMIPH ³
W09-505	Type A FP strain	+	TMIPH
SM101	Electroporatable derivative of type A FP strain NCTC8798, carries chromosomal <i>cpe</i> gene	+	T. Shimizu
MY15	SM101 <i>cpe</i> ::intron	–	This study
MY15(pJRC200)	MY15 complemented with pJRC200	+	This study
F4969	NFB disease type A strain, carries plasmid-borne <i>cpe</i> gene	+	[21]
MRS4969	F4969 <i>cpe</i> ::catP	–	[21]

¹Japan Collection of Microorganisms.

²National Collection of Type Cultures.

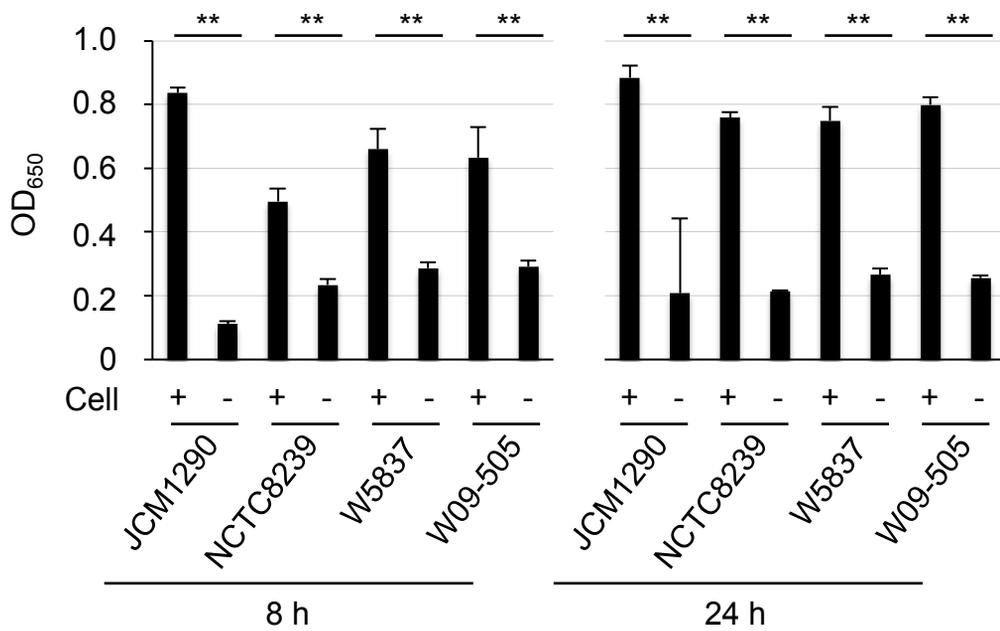
³Tokyo Metropolitan Institute of Public Health.

Table 2. Primers

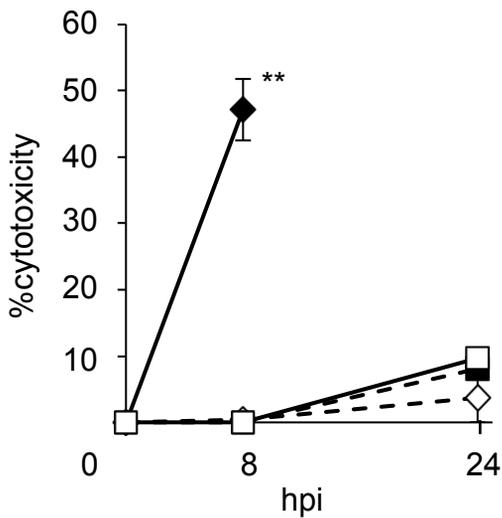
Primer name	Direction	Sequence	Ref.
PrMY44	16S rRNA-F	5'-CGCATAATGTTGAAAGATGG-3'	[55]
PrMY45	16S rRNA-R	5'-CCTTGGTAGGCCGTTACCC-3'	[55]
PrMY162	pfoA-F	5'-ATCCAACCTATGGAAAAGTTTCTGG-3'	[53]
PrMY163	pfoA-R	5'-CCTCCTAAAACCTACTGCTGTGAAGG-3'	[53]
PrMY8	IBS_195/6a	5'-AAAAAAGCTTATAATTATCCTTAACCTGCT TCATTGTGCGCCCAGATAGGGTG-3'	
PrMY9	EBS1d_195/6a	5'-CAGATTGTACAAATGTGGTGATAACAGAT AAGTCTTCATTAGTAACTTACCTTTCTTTGT-3'	
PrMY10	EBS2_195/6a	5'-TGAACGCAAGTTTCTAATTTTCGATTCAGGT TCGATAGAGGAAAGTGTCT-3'	
PrMY17	EBSuniversal	5'-CGAAATTAGAACTTGCGTTCAGTAAAC-3'	
PrMY20	cpe195/6-F	5'-AAGGAGATGGTTGGATATTAGG-3'	
PrMY21	cpe195/6-R	5'-ACCAGCAGTTGTAGATACAGATC-3'	
PrMY3	intron-R	5'-GTGTTTACTGAACGCAAGTTTCTAATTCG GT-3'	

Figure 1

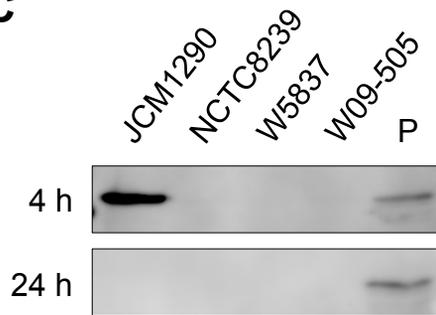
A



B



C



D

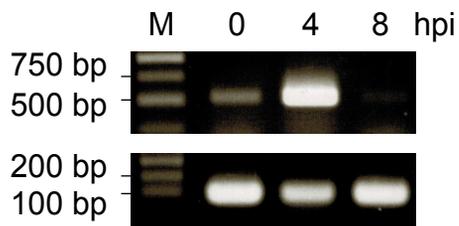


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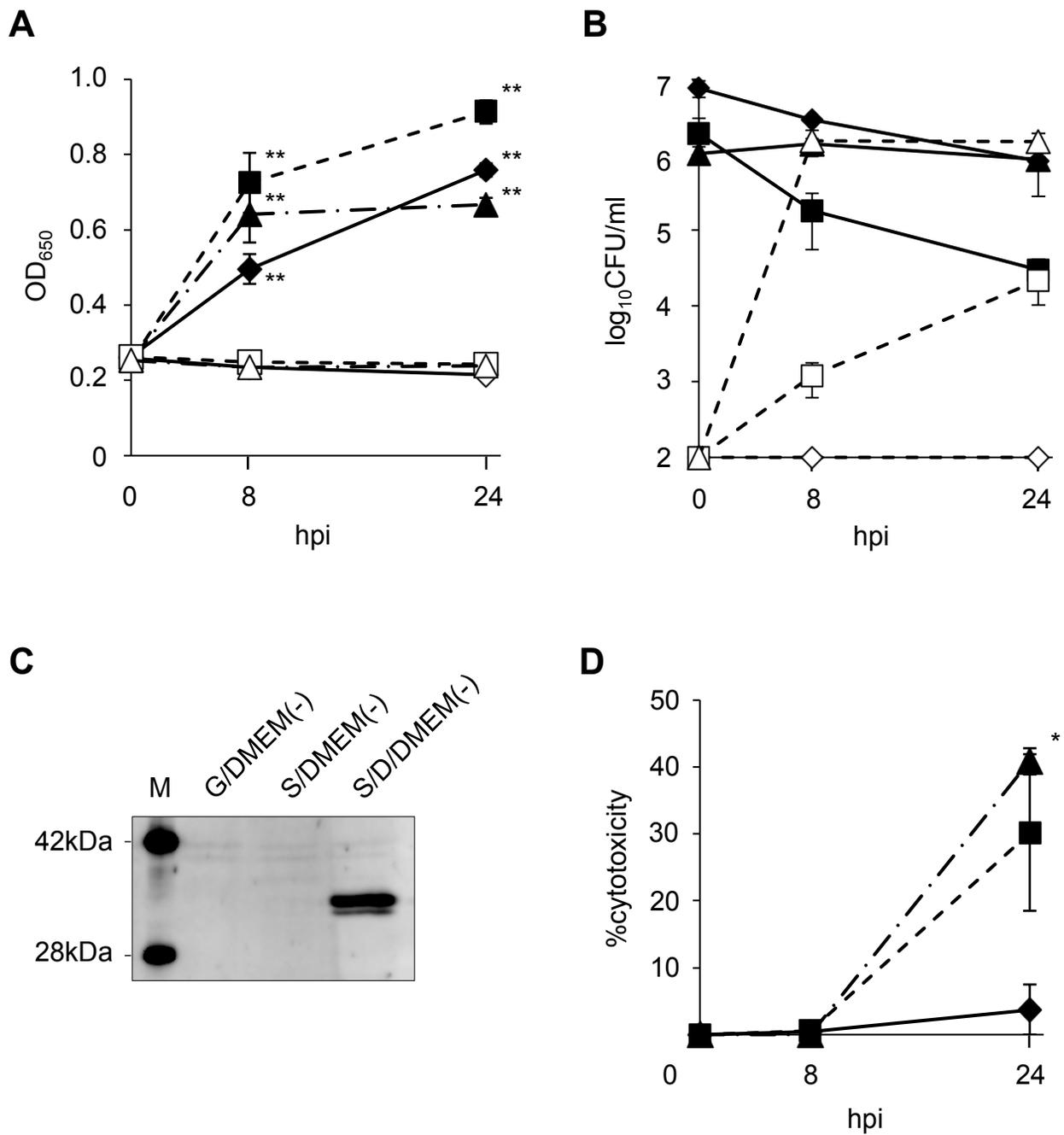
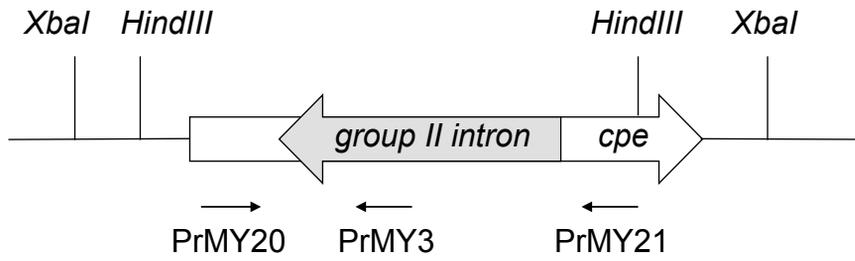
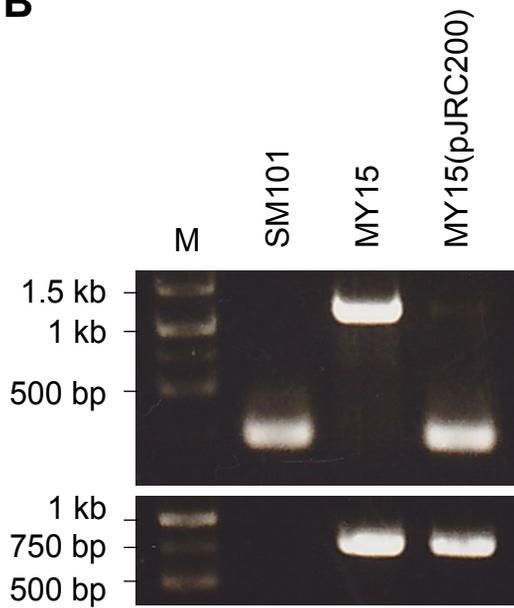


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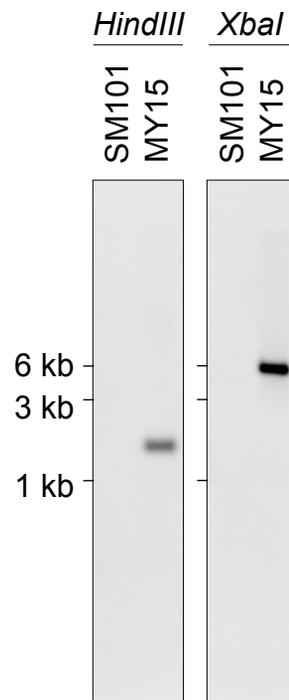
A



B



C



D

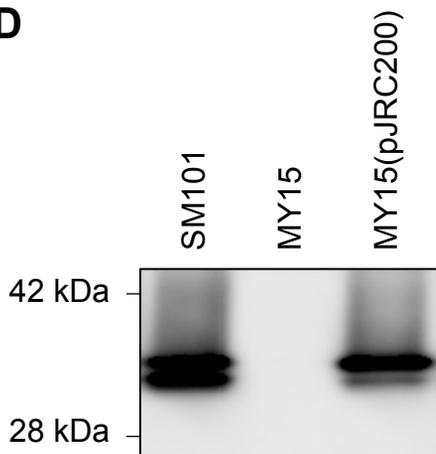


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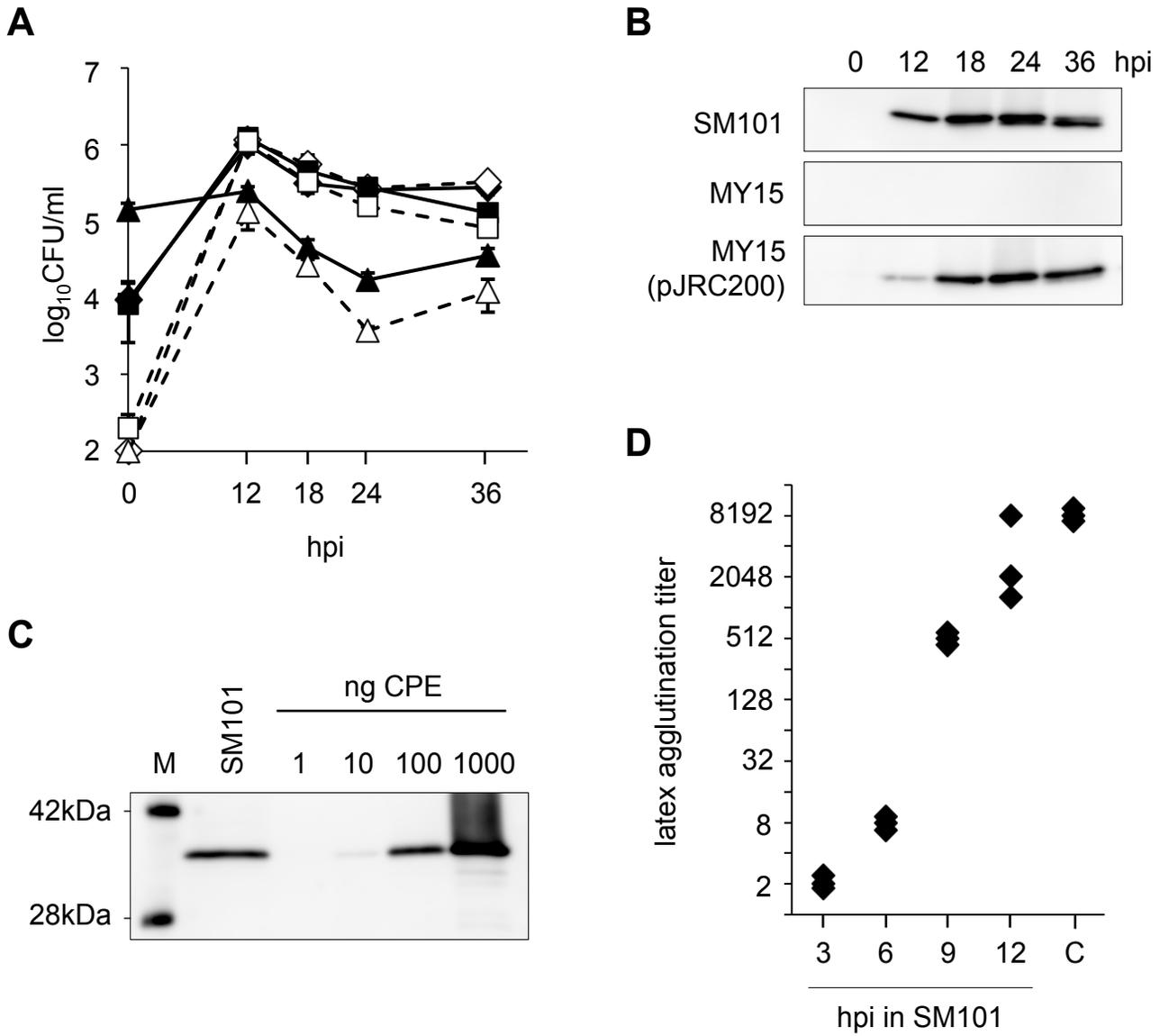


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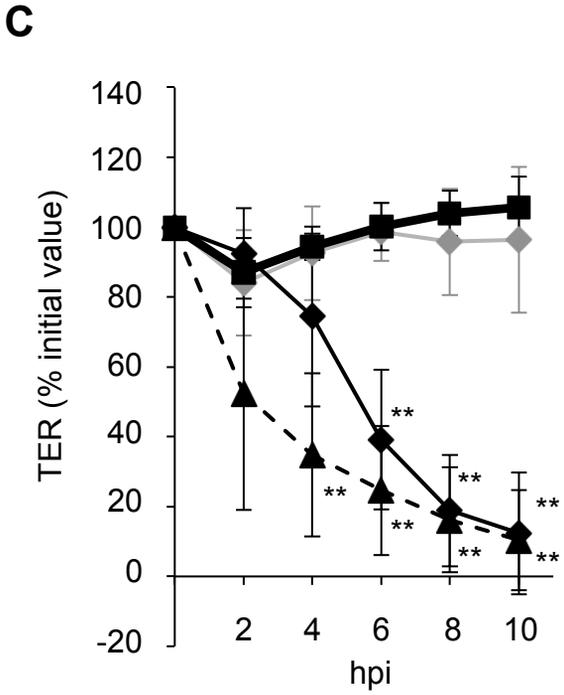
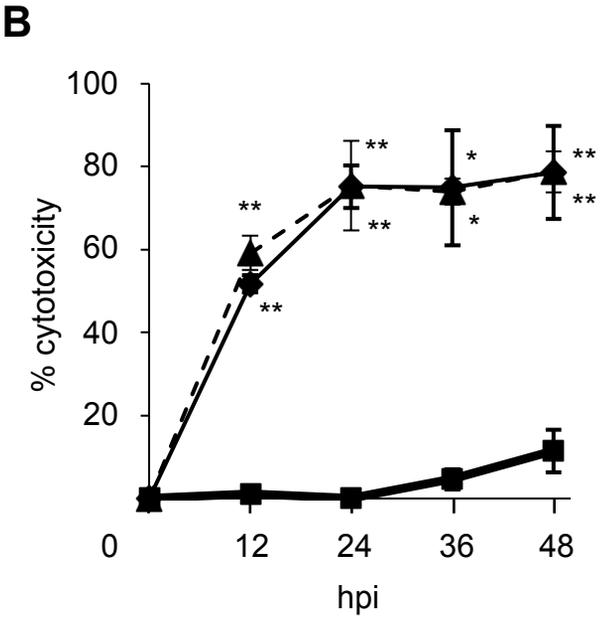
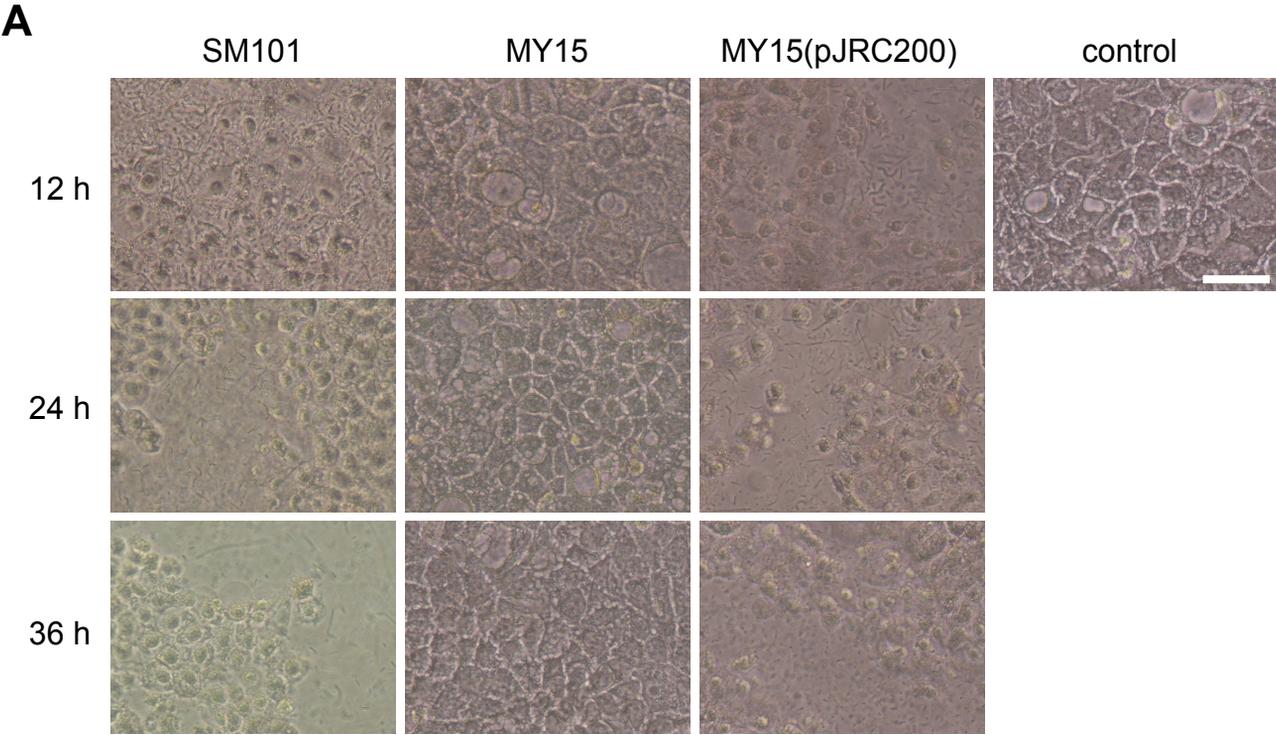


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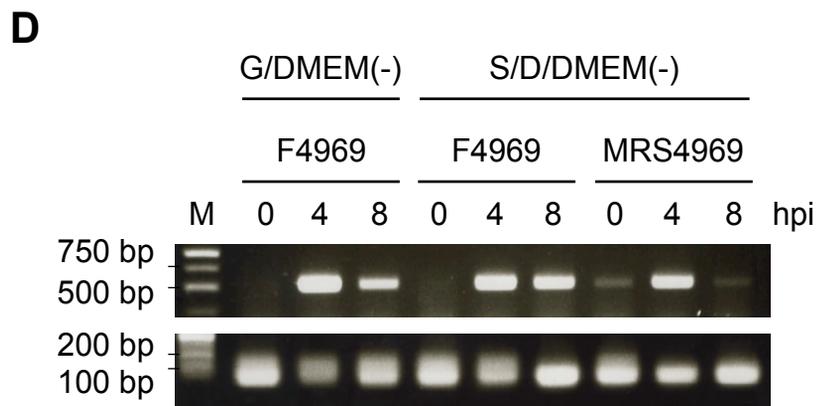
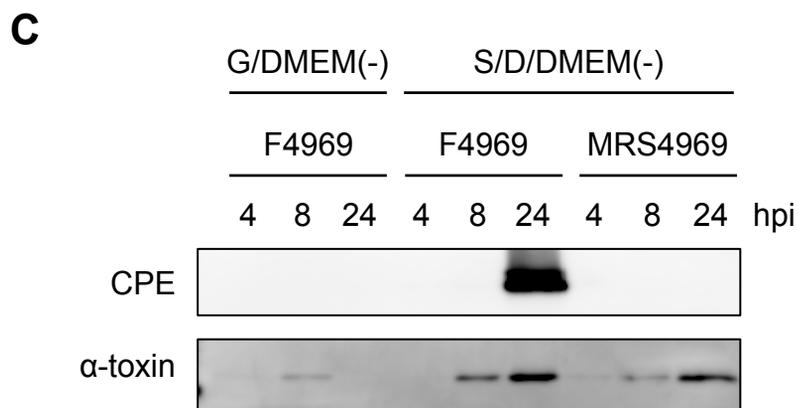
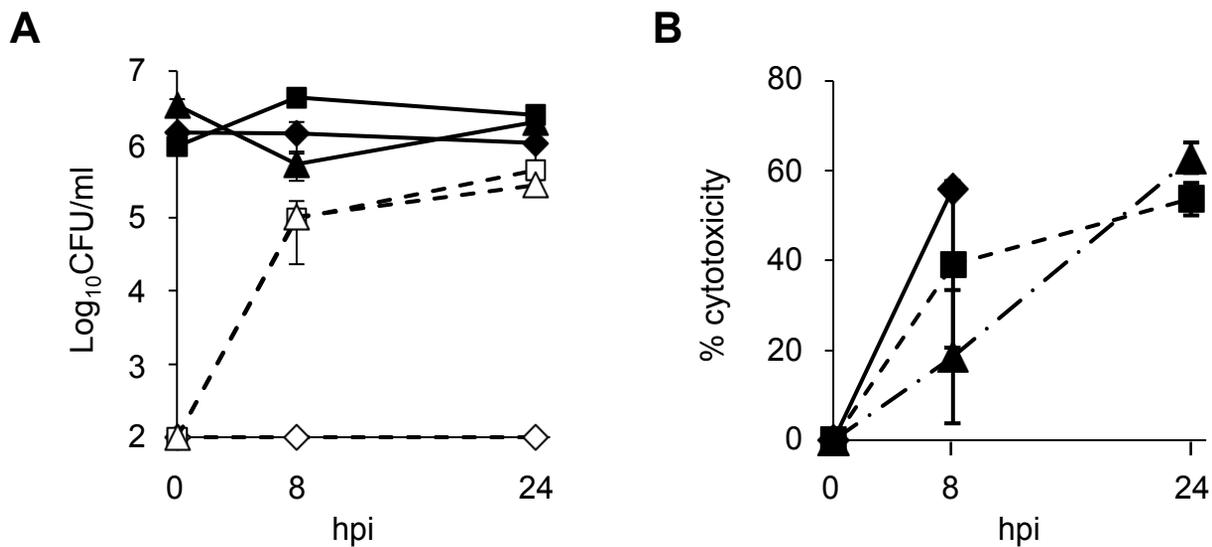
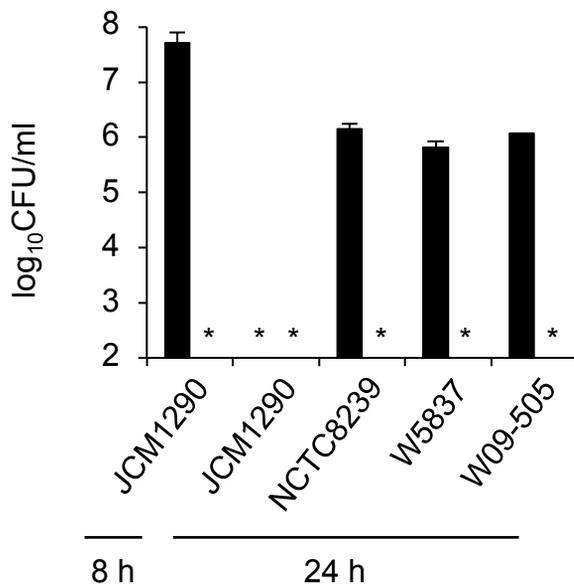


Figure S1

A



B

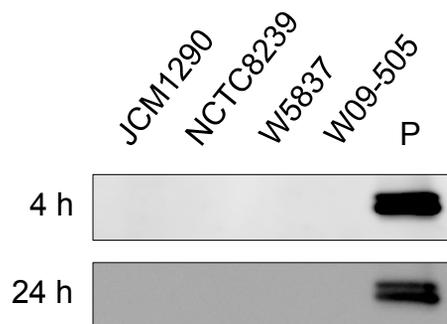


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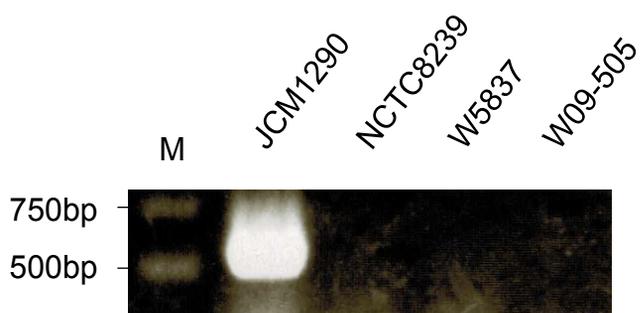
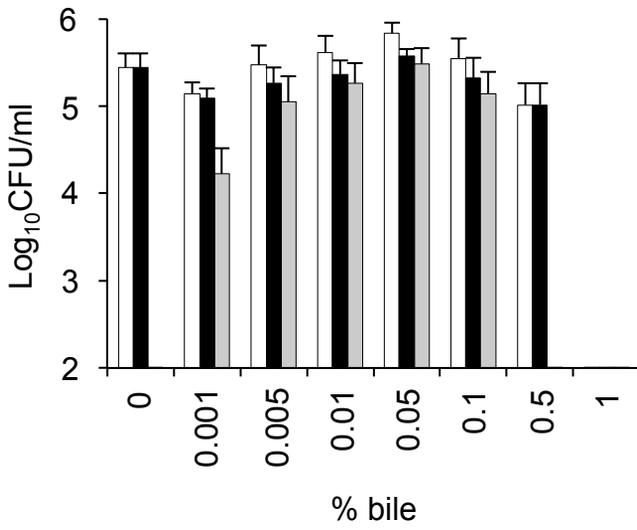
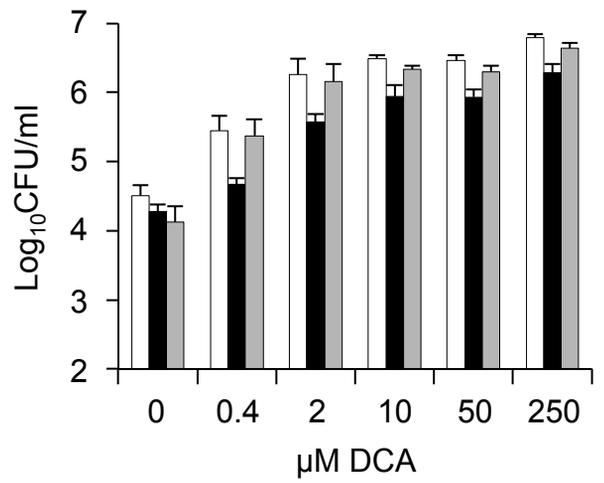


Figure S3

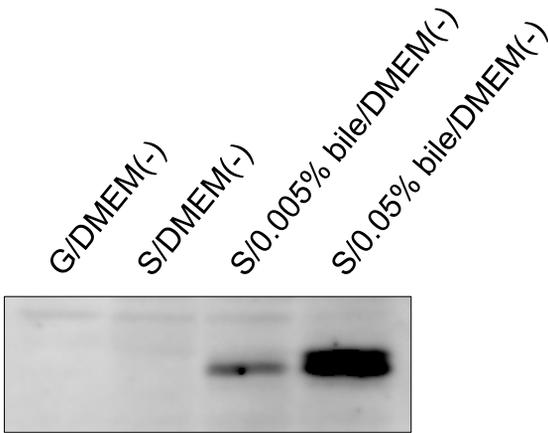
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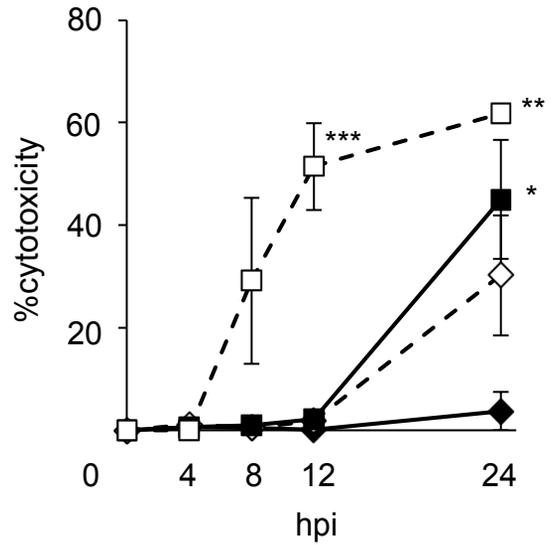
B



C



E



D

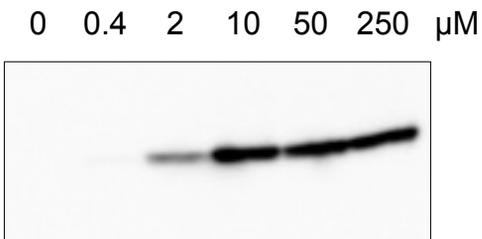
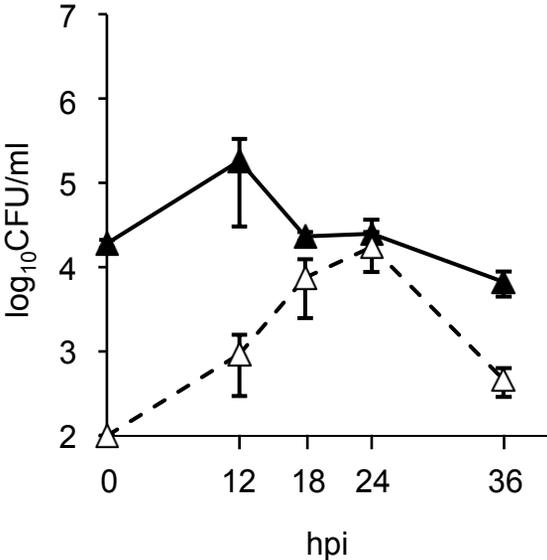
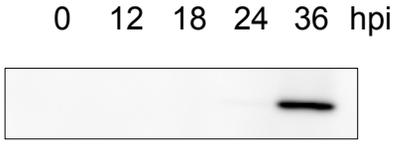


Figure S4

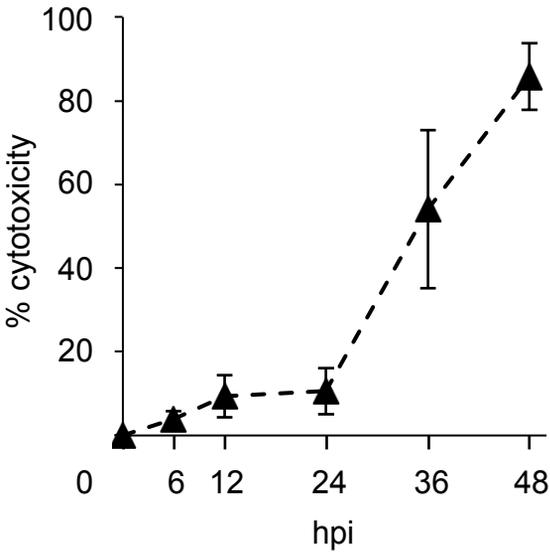
A



B



C



D

